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(54) **Title:** PROCESS FOR OBTAINING STEM CELLS

(57) **Abstract**

A method of obtaining a stem cell is described, comprising obtaining a somatic cell, which cell contains a gene coding for a marker which is differentially expressed in (a) desired stem cells and (b) cells other than desired stem cells; obtaining an enucleated oocyte; transferring the nucleus of the somatic cell into the enucleated oocyte to form a transgenic oocyte; culturing the transgenic oocyte to produce a culture of cells derived from the transgenic oocyte and identifying a cell of the culture that expresses the marker, thus indicating that the nucleus of that cell has been reprogrammed. Also described are assays for factors that can reprogram nuclei and stem cells obtained directly or indirectly using the nuclear reprogramming methods described.

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PROCESS FOR OBTAINING STEM CELLS

This invention relates to nuclear reprogramming of somatic cells, to a method of obtaining stem cells from nuclear transplanted embryos, and relates in particular to a method of obtaining embryonic stem (ES) cells. It also relates to pluripotent cells and to assays for reprogramming of somatic cell nuclei into pluripotent cell nuclei, and to assays for factors capable of reprogramming a somatic cell nucleus.

ES cells are pluripotent stem cells and can be used to generate a large range of somatic tissues such as haematopoietic stem cells, neural precursor cells and skin cells. The therapeutic value of being able to generate human embryonic stem cells *in vitro* would be considerable. Dominko et al have suggested bovine oocyte cytoplasm can successfully reprogram somatic nuclei from different mammalian species and initiate early embryonic development. It is thus hoped that ES cells will be obtainable from a wide range of mammalian embryos and will show great value in the development of transgenic animals. Many have claimed obtaining ES cells, but it has hitherto not been possible to prove that ES cells have in fact been obtained other than from the mouse, because they have until now been isolated on the basis of morphology. This is an unreliable test as embryonic structures derived from non-ES cells look virtually identical to embryonic structures that do contain ES cells.

Transplantation in mammals is presently hampered by the problems associated with rejection. When foreign tissues are introduced into a host its immune response is to attack what is perceived as invading tissue. This severe and life threatening response can be prevented by the use of immunosuppressant drugs, however, this leaves the host open to opportunistic infection. The immune response is not elicited when the host's own tissues are implanted. It would therefore be of advantage in transplantation if tissues that were

genetically identical to the host's could be generated.

Embryonic development in mammals commences with a series of cleavage divisions that generates equivalent totipotent cells. Maternal factors deposited in the oocyte dictate a characteristic number and timing of cleavages for each species. Activation of the zygotic genome occurs during the cleavage process. This is followed by the divergence of two distinct cell lineages, an outer epithelial cell layer, the trophectoderm, and an internal cluster of cells, the inner cell mass (ICM). The trophectoderm subsequently contributes the embryo-derived component of the placenta, whereas the ICM is the founder of the embryo proper. The ICM is pluripotent in that it gives rise to all foetal cell types, including the germ cells, and also to some extraembryonic tissues. Pluripotential cell lines, embryonic stem (ES) cells, can be derived by direct culture of ICM or its immediate pluripotent successor epiblast (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1996).

Once isolated the pluripotent stem cells can be cultured in the presence of particular growth factors and other signalling molecules that induce their differentiation into particular cell types. Particular tissue types are needed in different medical diseases and conditions. Haematopoietic stem cells could be used to treat individuals who are suffering from leukaemia and have been subject to loss of their own haematopoietic cells due to chemotherapy. Neural progenitor cells could be used to treat individuals suffering from neurodegenerative diseases such as Alzheimer's disease or Parkinsonism. Skin cells could be generated that are suitable for grafts in cases where an individual has suffered severe burns or scarring. Thus there is great therapeutic potential in being able to generate pluripotent stem cells that are genetically identical to a patient.

It is desirable to devise a further method by which human pluripotent stem cells can be made. It is in addition desirable to be able to make pluripotent stem

cells from a given individual. It is also desirable to be able easily to identify which cells in the early embryo are undifferentiated pluripotent stem cells. Furthermore, it would be advantageous to be able to isolate these pluripotent stem cells and then induce them to differentiate into a particular cell type. Finally, it is desirable to have an assay which is able to identify those factors that are necessary to maintain an undifferentiated state in the ES cell.

Nuclear replacement in oocytes is used to produce embryos and live offspring from somatic cells. This cloning procedure enables the production of genetically identical tissues or individuals, but the efficiency of current procedures is very low and only a small percentage of nuclear transfer embryos give viable offspring. It is likely that this is due in part to incomplete reprogramming of the donor cell nucleus. International patent publications WO-97/07668 and WO-97/07669 describe a method of transferring the nucleus of a somatic cell to a recipient cell, which could be an oocyte. Both WO-97/07668 and WO-97/07669 teach that the donor cell nucleus must be in a quiescent state for successful embryogenesis and subsequent implantation to occur. It would be advantageous to be able to identify which of the embryos produced by nuclear transplantation contain pluripotent cells prior to further culturing or implantation into a host mother.

The present invention aims to provide stem cells and an improved method for obtaining stem cells. It also aims to provide an improved method for obtaining human ES cells including transgenic human ES cells. The present invention further aims to provide an assay for factors capable of reprogramming somatic cell nuclei to a totipotent state, and an assay for identification of embryos or embryonic structures containing ES cells.

The present invention provides a combination of transplantation of a nucleus from a somatic cell into an enucleated oocyte with identification of daughter cells of the oocyte that express a gene characteristic of a desired stem cell. In preferred embodiments of the invention, cells expressing a gene

characteristic of embryonic stem cells are identified.

Accordingly, a first aspect of the invention provides a method of obtaining a stem cell of a predetermined species, comprising:-

obtaining a somatic cell of the predetermined species, which cell contains a gene coding for a marker which is differentially expressed in (a) desired stem cells, and (b) cells other than desired stem cells;

obtaining an enucleated oocyte;

transferring the nucleus of the somatic cell into the enucleated oocyte to form a transgenic oocyte;

culturing the transgenic oocyte *in vitro* to produce a culture of cells derived from the transgenic oocyte; and

identifying those cells of the culture that express the marker and, optionally, selectively propagating them.

It is a particular advantage of the invention that it facilitates the identification of embryos, and embryonic structures that will successfully go to term, and form adults. The high failure rate of prior art methods, in which as low as 1 per cent or less of embryos are viable has hitherto rendered this technology highly unreliable. The method of the invention may thus further comprise culturing the transgenic oocyte to form an embryo or embryonic structure, and determining whether the cells of the embryo or embryonic structure express the marker gene. Pluripotent cells are known to form the inner cell mass of the embryonic structure, and the method typically comprises culturing the transgenic oocyte to form an embryo or embryonic structure comprising an inner cell mass and trophectoderm cells, and determining whether the cells of the inner cell mass express the marker gene.

The somatic cell containing the marker gene may be obtained by introducing a transgene into a somatic cell, or by isolating a somatic cell containing the transgene from a transgenic animal. The invention thus encompasses first and descendant generations of transgenic stem cells. For generation of first generation transgenic stem cells, it is especially preferred that, starting with a somatic cell, a transgene is introduced into the somatic cell before the subsequent step of nuclear transfer into an enucleated oocyte. The somatic cell can be obtained via any standard biopsy procedure.

A key component of the present invention technology is the use of markers for ready identification of desired stem cells. In a specific embodiment of the invention, cells that are pluripotent stem cells express green fluorescent protein (GFP). Using UV light, visual inspection allows sorting of the cells or embryos so that non-viable ones can be discarded.

In one embodiment of the invention, the marker is a selectable marker wherein cells not expressing the selectable marker can be selectively killed or ablated in culture. For example, the selectable marker may be antibiotic resistance, such as a gene coding for neomycin resistance or G418 resistance.

The isolation and propagation of pluripotent ES cells is facilitated by elimination of differentiated cells. This is suitably achieved by expressing a selectable marker under control of regulatory sequences of the ICM marker gene, *oct4*, or of an Oct-4 responsive gene such as *fgf4*, or the gene for an Oct-4 co-factor such as Sox2, or of any other gene that is expressed selectively in ICM and epiblast cells. The selectable marker can be integrated into the endogenous gene by homologous recombination or may be inserted in a transgene construct. In addition a conditional immortalising gene can be introduced into a stem cell-specific locus to facilitate expansion of the pluripotent cells. A key indicator of functional reprogramming and a prerequisite for foetal development should be formation of a pluripotential ICM/epiblast that expresses Oct-4 in most or all cells, as it is known that Oct-4 is essential for development of

pluripotent cells in the mammalian embryo. Such embryos will give a high rate of post-implantation development and can be used as a source of ES cells.

An alternative is for the marker to be a reporter gene, wherein cells expressing the marker can be identified visually in culture. One suitable reporter is one which codes for a product that fluoresces in a particular light, such as GFP in UV light as mentioned or luciferase. Another is β-galactosidase which gives a blue colour with Xgal or a fluorescent signal with fluorescein digalactoside (FDG). It is particularly convenient that all can be used with the light microscope, so simple techniques are sufficient to identify the desired cells. The stains are preferably vital stains, in that they do not require fixing - killing of the stained tissue.

In use of the method of a specific embodiment of the invention, a transgene is introduced into a plurality of human somatic cells, the transgene containing a promoter active exclusively in embryonic stem cells and directing expression of GFP or of GFP fused to the stem cell specific gene product. The somatic cell nuclei are then each transferred into an enucleated pig oocyte. The oocytes are then cultured to form embryonic structures and these are subjected to illumination by UV and inspection under a microscope. Those few that show fluorescence within the ICM can be easily identified and isolated. From that point onwards the isolated embryonic structures can be further cultured *in vitro* or implanted. The use of the marker hence eases discarding the non-viable embryos that previously would have had to be identified on the basis of inability to implant, inability to go to term or subsequent morphological change indicating they are not in fact viable embryos.

A further alternative is for the transgene to code for an immortalising gene. This aids maintenance of a long term culture of embryonic stem cells obtained by the method.

In use of the method of a further specific embodiment of the invention, a

transgene is introduced into a plurality of human somatic cells, the transgene containing a promoter active exclusively in embryonic stem cells and directing expression of resistance to neomycin. The somatic cell nuclei are then each transferred into an enucleated cow oocyte. The oocytes are then cultured to form embryos from which cells which appear to be pluripotent stem cells are isolated. These cells are cultured in the presence of G418 and thus only those that are undifferentiated ES cells are able to survive.

In use of the method of a specific embodiment of the invention, a transgene is introduced into a plurality of human somatic cells, the transgene containing a promoter active exclusively in neuronal stem cells and directing expression of a selectable marker. The somatic cell nuclei are then each transferred into an enucleated recipient cell. The recipients are then cultured and these are subjected to selection. Those few that survive can be easily identified and isolated. From this point onwards the isolated neuronal stem cells can be further cultured *in vitro*. The use of the marker hence eases discarding the non-neuronal stem cells. In embodiments of the invention in which the aim is to obtain target stem cells other than ES cells, such as in the above embodiment for obtaining neuronal stem cells, the recipient is either an oocyte or preferably a target stem cell of a different individual. Thus an enucleated neuronal stem cell is used as the recipient for a transplanted somatic nucleus. Thus, to obtain neuronal stem cells, a plurality of somatic cells is first genetically manipulated to introduce a selection/reporter gene such as βgeo under control of the regulatory elements of sox1, sox2 or sox3, genes that are expressed uniformly in neuroepithelial precursor cells. For example, a selection/reporter gene, βgeo, is integrated by homologous recombination into the sox2 gene, which is expressed uniformly in precursor cells in the neural plate and neural tube. A transgene containing this construct is introduced into somatic cells, and application of G418 after nuclear transplantation results in the efficient isolation of viable βgeo-positive cells. These cells express markers of neuroepithelial precursor cells. They can be propagated and will differentiate efficiently into networks of neuron-like cells that express a variety of neuronal

markers. Thus, an in vitro system for genetic and molecular dissection of mammalian neural differentiation is provided by the invention, and also a route for the production of pure populations of normal or genetically modified neural precursors and neurons for functional studies including transplantation. The CD34, CD44 and SCL genes are suitable for obtaining haematopoietic progenitors, and the Nkx2.5 or GATA-4 gene for cardiac progenitors. For generating myogenic progenitors, MyoD or myf-5 are suitable.

It is an additional option that the method of the invention further comprises introducing into the somatic cell a selectable marker and a reporter marker, both expressed in the desired stem cells. This enables the visual identification and isolation of viable embryos, which can then be cultured under selection for the subsequent isolation of ES cells. The resultant culture contains stem cells that are genetically identical to the original donor somatic cell, except for the presence of the transgenes and of recipient cytoplast mitochondria. Use of excision sites flanking the transgenes are suitable to enable removal of the transgenes, such as by site specific recombinases.

The technology of the present invention is of application to all mammalian cell types, and to obtaining embryonic stem cells of a species for which there is a low (or no) availability of recipient oocytes. It is preferred that the somatic cell is a human cell, and surprisingly the invention provides for creation of human embryonic stem cells from a non-human oocyte - pig or cow oocytes are used in the recited examples below, as they are readily available.

Once embryonic stem cells are isolated, they can be used as a resource for deriving progenitor cells of a selected lineage. As described and claimed in co-pending GB patent application 9807935.3 filed on 14th April 1998, neuronal, haematopoietic progenitors, cardiac and other progenitors are obtainable using a further transgene expressed substantially only in the desired progenitors, and also cells of a selected differentiated non-stem cell lineage are similarly obtainable.

A second aspect of the invention provides a method of obtaining stem cells comprising:-

obtaining a somatic cell from an individual which cell comprises a transgene which is differentially expressed in (a) desired stem cells, and (b) cells other than desired stem cells;

transferring the nucleus of the somatic cell into an enucleated oocyte;

culturing the oocyte to form an embryo or an embryonic structure; and

determining whether cells in the resultant embryo or embryonic structure are expressing the transgene.

The method is preferably for obtaining mammalian embryonic stem cells, wherein the transgene is differentially expressed in (a) cells of the inner cell mass, and (b) cells other than cells of the inner cell mass, and the method comprises culturing the oocyte to form an embryo or embryonic structure and determining whether the cells of the inner cell mass express the transgene. It is particularly preferred that the transgene is expressed substantially only in cells of the inner cell mass. It is an option that blastocysts are developed in vivo, e.g. in ligated oviducts.

The transgene can code for a selectable marker, wherein cells not expressing the selectable marker can be selectively killed or ablated in culture; or the transgene can code for a reporter marker which fluoresces when illuminated with light of a certain wavelength, and wherein cells expressing the reporter marker can be identified by their fluorescence in the presence of the light. Other preferred and alternative features of the first aspect of the invention apply equally to the second aspect, and further aspects, of the invention.

A third aspect of the invention provides an assay for the effect of a factor on

a nucleus of a somatic cell, comprising:-

introducing the nucleus into an enucleated cell, wherein the nucleus comprises a marker that is differentially expressed in (a) a desired stem cell, and (b) a cell other than the desired stem cell;

introducing the factor into the cell;

maintaining the cell in *in vitro* culture; and

determining whether the cell or any of its daughter cells are expressing the marker, whereby expression of the marker indicates the somatic cell nucleus has been reprogrammed to be a nucleus of the desired stem cell.

By "reprogrammed", and derived terms, we mean that a nucleus from a cell with restricted developmental potential acquires greater developmental potential, e.g. that a unipotent nucleus from a differentiated somatic cell is thereby able to be directed to develop into a pluripotent nucleus of a stem cell, or that a multipotent nucleus from a somatic stem cell is thereby able to be directed to develop into a pluripotent stem cell nucleus. Preferably, the somatic cell nucleus is able to be reprogrammed such that when it is implanted into the enucleated oocyte it can facilitate the development of a normal embryo, which can then be implanted and go to term to form an adult animal or which can then be used for derivation of embryonic stem cells and, subsequently, differentiated cells if so desired.

An advantage of the assay is that it assists screening for a factor or factors that induce reprogramming of nuclei. Factors so identified can then be used as agents further to increase the reliability and efficiency of the technique. The assay is suitably performed using enucleated oocytes. The assay is also suitably performed using enucleated cells other than oocytes, and this has the advantage that the effect of the factor is assayed without interference from

any nucleus reprogramming factor that might be already coincidentally present, as might be the case if an oocyte is used. Fibroblasts are one suitable cell type.

For identification of factors to reprogram nuclei into pluripotential nuclei, the marker used in the assay is differentially expressed in (a) pluripotential stem cells, and (b) cells other than pluripotential stem cells.

The assay is suitable for assay of a library of factors, such as a library of factors obtainable by expression of a cDNA library obtained from mRNA extracted from the cytoplasm of an oocyte.

As an alternative to the above recited procedure, it is an option for the assay to be carried out without nuclear transfer, but nevertheless providing for identification of a factor that reprograms the nucleus of a somatic cell. Specifically, the assay may comprise:-

maintaining a culture of a cell, which cell comprises a marker differentially expressed in (a) a desired stem call and (b) a cell other than a desired stem cell;

introducing into the cell a factor to be assayed;

determining whether the cell or any of its daughter cells are expressing the marker, whereby expression of the marker indicates that somatic cell nucleus has been reprogrammed to be a nucleus of a desired stem cell.

This embodiment of the invention can conveniently be used for assay of an oocyte library to identify a factor that activates OCT 4 in, say, a fibroblast cell.

It is also desirable to assay for embryos that have the potential to go to term, and for embryonic structures that contain pluripotent cells. Accordingly, a

fourth aspect of the invention provides an assay to determine whether an embryo or embryonic structure contains embryonic stem cells, comprising:-

introducing into an enucleated oocyte the nucleus of a somatic cell comprising a marker differentially expressed in (a) embryonic stem cells, and (b) cells other than embryonic stem cells;

obtaining therefrom an embryo or embryonic structure; and

determining whether cells of the embryo or embryonic structure express the marker.

In an embodiment of the invention, the assay comprises introducing into the somatic cell a transgene containing a sequence coding for the marker under control of a promoter activated substantially only in epiblast cells. One specific example is the *oct4* promoter, or a promoter activated in cells expressing the *oct4* gene.

After introduction of the transgene, there will be some somatic cells that are stably transfected, but many that are not. In a preferred embodiment of the invention the transgene also contains or is cotransfected with a sequence coding for a second marker under control of a promoter activated in substantially all cells, and the assay comprises determining whether the transgene has been stably integrated into the nucleus by determining whether the somatic cell expresses the second marker.The beta-actin promoter is one example. In this way, stable transfectants can be identified before determination of those that express the first marker, and optionally before the step of nuclear transplantation. Preferably, the second marker is a selectable marker, enabling selection of stable transfectants. The second marker may be flanked with excision sites to enable its removal prior to further manipulation.

In fifth to twelfth aspects of the invention there is provided the cells obtainable

by nuclear transfer according to the invention, namely:-

a human embryonic stem cell derived from a human oocyte;

a human embryonic stem cell derived from a non-human oocyte;

a human embryonic stem cell derived from a non-human mammalian oocyte;

a human embryonic stem cell derived from a pig or cow oocyte;

a human stem cell derived from a human oocyte;

a human stem cell derived from a non-human oocyte;

a human stem cell derived from a non-human mammalian oocyte; and

a human stem cell derived from a pig or cow oocyte;

The invention still further provides a stem cell for transplantation into a first human and being immunologically compatible with the first human, derived from an oocyte of a different human; and an embryonic stem cell of a first mammalian species, derived from an oocyte of the same species or of a species other than the first species, and comprising a transgene coding for a selectable marker which is substantially only expressed in the embryonic stem cell; and a multipotential cell that expresses a transgene which identifies its undifferentiated state, which cell is genetically identical, apart from the presence of the transgene, to an adult animal.

The invention yet further provides a method of obtaining a human embryonic stem cell, suitable for derivation of human stem cells or human somatic cells

for transplantation into a first human individual, comprising introducing into an oocyte other than of that first individual the nucleus from a somatic cell of that first individual. The human embryonic stem cell preferably contains a transgene coding for a selectable marker which is substantially only expressed in the human embryonic stem cell.

The invention again further provides a method of reprogramming a somatic nucleus into a pluripotent nucleus, comprising:-

identifying a factor that induces reprogramming of the nucleus, by carrying out the assay according to the third aspect of the invention;

obtaining a somatic cell nucleus;

obtaining an enucleated cell;

transferring the somatic cell nucleus into the enucleated cell to form a recombinant cell;

introducing the factor into the recombinant cell.

The invention yet again further provides a method of reprogramming a somatic nucleus into a pluripotent nucleus, comprising:-

identifying a factor that induces reprogramming of the nucleus, by carrying out the assay according to the third aspect of the invention;

obtaining a somatic cell, and introducing the factor;

obtaining an enucleated cell;

transferring the nucleus of the somatic cell into the enucleated cell to

form a recombinant cell;

culturing the recombinant cell *in vitro*.

In these above two methods, it is optional to introduce into the somatic cell a gene coding for a marker which is differentially expressed in (a) desired totipotent cells, and (b) cells other than desired totipotent cells; and determine whether cells of the embryo are expressing the marker. Even though a reprogramming factor has been identified by the assay, using these optional steps facilitates isolation of reprogrammed nuclei, which nuclei can then be propagated if desired and can be introduced into enucleated oocytes.

In use of a typical method of the invention a pluripotent cell specific selectable marker and/or reporter and/or immortalising gene is introduced into a somatic cell culture, for example a culture of foetal or adult fibroblasts, and a nucleus from a cell harbouring said marker(s) is transferred into an enucleated oocyte or other suitable cytoplast. Visualisation of, or selection for, expression of the stem cell specific marker then allows detection of, or selection for, successful reprogramming. Pluripotential stem cell cultures are isolated and propagated under selection for marker gene expression and /or influence of the immortalising gene.

In a specific embodiment of the invention, described in an example below, the selectable marker/reporter is introduced under control of the regulatory elements of the gene encoding the essential pluripotent cell-specific transcription factor Oct-4 (Mountford *et al.*, 1994). In another embodiment, the selectable marker is placed under regulatory control of an Oct-4 responsive sequence such as the *fgf4* gene or of a stem cell restricted co-factor of Oct-4 such as Sox2, or of another ICM/epiblast specific gene. The selectable marker preferably encodes both a reporter gene function, such as β-galactosidase or GFP, and a drug resistance function. This dual function can be achieved in the form of a fusion gene, such as the *lacZ/neo* fusion βgeo, or of a dicistronic

construct incorporating an internal ribosome entry site (IRES), for example *gfp*IRES*pac*. In an alternative embodiment the transgene construct may incorporate an immortalising gene to facilitate subsequent growth of the pluripotent stem cells. This may be included instead of, or in addition to, the selectable marker/reporter. One such immortalising gene is that encoding SV40 large T protein. The immortalising gene is rendered conditional, for example by using a temperature or hormone-regulated variant and/or by incorporation of target sequences for site-specific recombination or endonuclease degradation. A combination of reporter, selectable marker and immortalising gene can be introduced either in a single construct using gene fusions and/or IRES elements, or by introduction of separate transgenic constructs.

The POU transcription factor Oct-4 is expressed in all pluripotent cells in the ICM and epiblast and in germ cells. Oct-4 is absent from mature trophectoderm and all other differentiated cell types. The inventors have discovered that Oct-4 is essential for the establishment of the pluripotential identity of the ICM. In blastocyst stage embryos in which the *oct-4* gene has been inactivated, internal cells fail to develop ICM character and are diverted into the trophectoderm lineage. Oct-4 function is also continuously required to maintain pluripotency of ES cells. Inactivation of the *oct-4* gene in established ES cell cultures results in a failure of self-renewal and terminal differentiation. Oct-4 controls the expression of multiple target genes. Genes that are positively regulated by Oct-4, such as the gene for fibroblast growth factor-4 (FGF-4), exhibit a similar restricted pattern of expression in ICM and epiblast. Oct-4 functions in collaboration with co-factors such as the HMG-box transcription factor Sox2 and the E1A-like activity whose expression may be similarly restricted in the early embryo.

The selectable marker is preferably placed under transcriptional control of Oct-4 regulatory sequences either by homologous integration into the *oct-4* gene or random integration of a transgene construct. In the example the selectable marker is integrated into the 3'UTR of the *oct4* gene and is preceded by an

EMCV IRES sequence inserted immediately 3' to the *oct4* termination codon. Alternatively, the selectable marker is integrated either directly at the ATG start codon, or in-frame within the Oct-4 coding sequence, or in any frame within the Oct-4 coding sequence in which case it should be preceded by a translational termination codon in the *oct-4* reading frame and an IRES element. Optionally, the transgene may be flanked by recognition sequences for a site specific recombinase such as Cre or Flp to enable subsequent deletion, or may contain a site for a rare restriction site endonuclease such as I-*sce*I. For introduction into somatic cells in culture the transgene construct will normally contain or be co-transfected with a construct containing an independent selectable marker to enable isolation of stable transfectants. This selectable marker should be distinct from that inserted into the *oct-4* gene and must be under the transcriptional control of a promoter active in the target somatic cell, for example the promoter of a ubiquitously expressed gene such as *β-actin*. The independent selectable marker may be flanked by recognition sequences for a site specific recombinase to allow subsequent excision. These sites should be for a different recombinase than sites flanking the stem cell-specific marker transgene to avoid excision of the latter.

The transgene construct may be introduced via pronuclear injection to generate a transgenic animal from which somatic cells can be isolated or is introduced directly into somatic cells *in vivo* or in culture by microinjection, viral transduction, lipofection, electroporation, calcium phosphate co-precipitation or other method. Integrity of the transgene and integration by legitimate or illegitimate recombination are determined by genomic DNA analysis. Where appropriate transient introduction of the appropriate site-specific recombinase is used to delete the independent selectable marker.

Nuclei from cells harbouring the transgene are transferred into enucleated oocytes or other suitable cytoplasts using established procedures (Campbell *et al.*, 1996; Wilmut *et al.*, 1997) or technical modifications thereof such as the use of microinjection rather than fusion. Recipient oocytes may be of the same

species as the donor cell or of a different species. Thus bovine or other animal oocytes may be used for reprogramming human somatic cell nuclei. Following nuclear transfer, activated reconstituted oocytes may be transferred to the reproductive tract of a primed female for development *in vivo* with subsequent recovery and examination at the blastocyst stage. *In vivo* development of nuclear transfer embryos could also be achieved by encapsulation and placement at a favourable ectopic site, e.g. in the reproductive tract or subcutaneously, in an animal of the same or different species. Alternatively embryos may be developed *in vitro* by culture intact to the blastocyst stage. Expression of the reporter may be determined by histochemical or immunohistochemical analysis of fixed specimens, but more normally is examined in live embryos by visualisation of vital fluorescent staining, either directly for GFP or using a fluorescent substrate for β-galactosidase.

Embryos showing specific and uniform staining in the ICM/epiblast are selected for transfer to recipients to produce embryos or live offspring. For derivation of stem cell lines, embryos may be explanted directly into culture. Alternatively the stem cell compartment may first be expanded *in vivo*, either by allowing implantation and a brief period of post-implantation development, and/or by transplantation of cleavage stage embryos, blastocysts or egg cylinders to an ectopic site such as the kidney capsule or testis of a syngeneic or immunocompromised host for teratoma development (Solter *et al.*, 1970). The embryo and recipient are not required to be of the same species for teratoma formation. Induction of human teratomas can be achieved in nude or SCID mice, for example. Cultures initiated from embryonic material or teratomas are maintained in the presence of the selection drug until continuously growing ES cells are established. Pluripotent EG cell cultures (Matsui *et al.*, 1992) may also be initiated from primordial germ cells following uterine transfer and implantation. Again, the cultures are carried out in the presence of selection agent to eliminate non-stem cells.

There now follows description of specific embodiments of the invention

illustrated by drawings in which:-

Fig. 1 shows an Oct4-βgeo expression construct according to the invention;

Fig. 2 shows an egg cylinder stage embryo, 6.5 dpc (upper panel) and blastocysts from a backcross mating (lower panel), illustrating expression of β-galactosidase in ICM and epiblast of transgenic embryos;

Fig. 3 shows cultured epiblast of a transgenic embryo, illustrating expression of β-galactosidase is maintained in culture;

Fig. 4. shows β-galactosidase staining in (A) morulae and (B) blastocyst stage Oct4-bgeo transgenic mouse embryos; and

Fig. 5. shows β-galactosidase staining in nuclear transfer mouse embryos following transfer of Oct4-bgeo somatic cell nuclei into non-transgenic recipient cytoplasts.

Example 1

Mouse genomic clones spanning the Oct-4 locus were isolated from a strain 129 genomic library and mapped by restriction analysis. A transgene construct was prepared comprising the distal and proximal 5′ enhancer elements, the entire structural gene, a IRESβgeo cassette inserted at the translation termination codon, the 3′ untranslated sequence and several kb of 3′ sequence (Figure 1).

Transgenic rats harbouring this transgene were generated by pronuclear injection. In these rats expression of the transgene faithfully reproduced expression of Oct-4. Thus β-galactosidase activity was present in the inner cell mass of blastocysts and the epiblast of early post-implantation embryos but was absent from extraembryonic lineages and from differentiating embryonic

germ layers (Figure 2). Post-gastrulation expression of β-galactosidase was confined to germ cells. Significantly also, β-galactosidase activity ie specific expression of the selectable marker was maintained in culture (Figure 3).

Having confirmed that the transgene contained appropriate regulatory sequences to dictate pluripotential cell-specific expression, it was modified for transfection into cultured somatic cells. As Oct-4 is not expressed in these cells, this required introduction of an independently expressed selection marker. A hygromycin resistance-thymidine kinase fusion gene (hph/tk) under transcriptional control of the mouse *pgk-1* promoter was introduced into the 3' end of the transgene, downstream of the untranslated region. The pgk-hph/tk cassette was flanked by *loxP*sites and the entire transgene fragment was flanked by *frt* sites.

The linearized construct was introduced into primary mouse embryo fibroblasts by electroporation and clonal isolates selected in the presence of hygromycin. Clones were assayed for ectopic activity of the Oct-4 transgene by plating aliquots in the presence of 200 μg/ml G418. Only those that failed to grow in G418 were studied further. Three such clones (I, II and III) with single copy integrations determined by Southern analysis were transiently transfected with Cre recombinase and selected in the presence of gancyclovir. Gancyclovir resistant colonies were screened by PCR to confirm Cre-mediated excision of the pkg-hph/tk cassette. Deletion of this cassette removes the potential for interference with activity of the Oct-4 transgene.

Resultant clones carrying the Oct-4βgeo transgene only were used for nuclear transfer into mouse, ovine and bovine enucleated oocytes. The oocytes were quiesced by serum-deprivation and nuclear transfer was carried out as described by Campbell et al (1996). In some cases a serial round of nuclear transfer was performed. After nuclear transfer the activated oocytes were cultured in embryo culture medium and monitored for cleavage, morula formation and blastocyst development. Embryos were fixed and stained for β-

galactosidase activity using Xgal. No staining was observed after nuclear transfer of clone II derivatives, presumably due to a non-permissive integration site. Derivatives of clones I and III in contrast gave ICM-specific staining in 30-50% of embryos that developed to the blastocyst stage, regardless of oocyte origin. Of these approximately half showed mosaic staining and half showed uniform staining in all ICM cells. Thus only around 25% of morphologically developed blastocyts show normal expression of the Oct-4 transgene.

The developmental capacity of Oct4βgeo expressing and non-expressing embryos generated after nuclear transfer into mouse oocytes was then compared. A vital fluorescent substrate for β-galactosidase (Molecular Probes) was used to visualise transgene expression in live embryos. Blastocysts were categorised as non-staining, mosaic ICM staining or uniform ICM staining and divided accordingly into three groups. Each group was transferred into the uteri of separate pseudopregnant mouse recipients. Mice were sacrificed 5 days later for analysis of implantation and development. The first two groups gave fewer than 50% implantations. High numbers of resorption sites and abnormal embryos were found in both cases. Morphologically normal embryo development was not observed after transfer of class I embryos and only in 1 in 10 implantations generated by class II embryos. In contrast, class III embryos gave greater than 50% implantation rates and the majority of embryos were normal. The analysis was then repeated and pregnancies allowed to go the term. Liveborn offspring were obtained from class III embryos only.

In order to isolate stem cell lines, nuclear transfer embryos were developed *in vivo* to the late blastocyst or early implantation stage. Epiblasts were microdissected (Brock and Gardner, 1997) and placed in culture in ES cell medium in the presence of G418. After several days the epiblasts were dissociated and replated. Expanding populations of undifferentiated stem cells were generated. Stem cell lines were also derived form embryos developed *in vitro* by immunosurgical isolation of the ICM at the blastocyst stage followed by microsurgical removal of the primitive endoderm and culture in ES cell

medium plus G418.

Fibroblast clones I and III in which the transgene was competent for reprogramming were also used to assay for factors that mediate reprogramming. Messenger RNA was prepared from oocytes, fractionated on a sucrose gradient, and microinjected directly into fibroblast nuclei. G418 selection was then applied to identify RNA pools containing reprogramming activity. cDNA expression libraries were prepared from positive pools and transfected into the fibroblasts to enable isolation of sequences encoding reprogramming factors.

Example 2

Mouse genomic clones spanning the Oct-4 locus were isolated from a strain 129 genomic library and mapped by restriction analysis. A transgene construct was prepared comprising the distal and proximal 5' enhancer elements, the entire structural gene, a IRES βgeo cassette inserted at the translation termination codon, the 3' untranslated sequence and several kb of 3' sequence (Figure 1).

Transgenic mice harbouring this transgene were generated by pronuclear injection. β-galactosidase activity was monitored by Xgal staining of fixed embryos and tissue and is evident in morulae and blastocysts stage pre-implantation mouse embryos (Figure 4). As expected, β-galactosidase activity was not detected in somatic cells including cumulus cell and foetal fibroblast nuclear donors.

Nuclei from somatic cells of transgenic mice were used for nuclear transfer. Enucleated and non-enucleated wild-type oocytes were used as recipient cytoplasts. In some cases a serial round of nuclear transfer was performed. After nuclear transfer the activated oocytes were cultured in embryo culture medium and monitored for cleavage, morula formation and blastocyst development. Embryos were fixed and stained for β-galactosidase activity

using Xgal. Staining corresponding to Oct4 expression was observed in approximately 50% of the nuclear transfer embryos at the eight-cell stage of development (Figure 5) confirming successful nuclear reprogramming. Embryos which did not show β-galactosidase activity failed to reprogram the Oct4-bgeo transgenic nucleus, or to functionally incorporate the transgenic nucleus and were therefore scored as compromised in developmental capacity.

Nuclear transfer embryos can be stained with the vital fluorescent substrate for β-galactosidase (Molecular Probes) to visualise transgene expression in live embryos. Embryos categorised as non-staining, mosaic or uniform staining can be divided according to nuclear status and associated developmental capacity based on the transgene expression profile.

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This invention thus enables increased efficiency of cloned and transgenic animal production by nuclear transfer, provides an assay for factors that mediate or suppress reprogramming, provides a generic method of ES cell derivation for mammals, and provides a method of establishing pluripotent stem cell cultures from human individuals of any age.

Claims

1. A method of obtaining a stem cell of a predetermined species, comprising:-

obtaining a somatic cell of the predetermined species, which cell contains a gene coding for a marker which is differentially expressed in (a) desired stem cells, and (b) cells other than desired stem cells;

obtaining an enucleated oocyte;

transferring the nucleus of the somatic cell into the enucleated oocyte to form a transgenic oocyte;

culturing the transgenic oocyte *in vitro* to produce a culture of cells derived from the transgenic oocyte; and

identifying a cell or those cells of the culture that express the marker.

2. A method according to Claim 1 comprising selectively propagating cells that express the marker gene.

3. A method according to Claim 1 or 2, comprising culturing the transgenic oocyte to form an embryo or embryonic structure comprising an inner cell mass and trophoectoderm cells, and determining whether the cells of the inner cell mass express the marker gene.

4. A method according to Claim 1, 2 or 3 wherein the marker is a selectable marker wherein cells not expressing the selectable marker can be selectively killed or ablated in culture.

5. A method according to Claim 4 wherein the selectable marker is

antibiotic resistance.

6. A method according to Claim 1, 2 or 3 wherein the marker is a reporter, wherein cells expressing the marker can be identified visually in culture.

7. A method according to Claim 6 wherein the reporter gene codes for a product that is excited by light, such as GFP or luciferase.

8. A method according to Claim 1, 2 or 3 wherein the marker is an immortalising gene.

9. A method according to any of Claims 1 to 8, comprising introducing into the somatic cell a selectable marker and a reporter marker.

10. A method according to any previous Claim wherein the gene coding for the marker can be removed from the nucleus of a stem cell obtained according to the method.

11. A method according to Claim 10, wherein the gene coding for the marker can be so removed using a site specific recombinase.

12. A method according to any previous Claim wherein the somatic cell is a mammalian cell.

13. A method according to Claim 12 wherein the somatic cell is a human cell

14. A method according to any previous Claim wherein the oocyte is a non-human cell.

15. A method according to any of Claims 1 to 13 wherein the oocyte is a human cell.

16. A method according to any previous Claim for obtaining embryonic stem cells.

17. A method according to any previous Claim, further comprising deriving progenitor cells of a selected lineage from the embryonic stem cells

18. A method according to Claim 17 for obtaining neuronal progenitors.

19. A method of obtaining stem cells comprising:-

obtaining a somatic cell from an individual, which cell comprises a transgene which is differentially expressed in (a) desired stem cells, and (b) cells other than desired stem cells;

transferring the nucleus of the somatic cell into an enucleated oocyte;

culturing the oocyte to form an embryo or an embryonic structure; and

determining whether cells in the embryo or embryonic structure are expressing the transgene.

20. A method according to Claim 19 for obtaining mammalian embryonic stem cells, wherein the transgene is differentially expressed in (a) cells of the inner cell mass, and (b) cells other than cells of the inner cell mass, and the method comprises culturing the oocyte to form an embryo or embryonic structure and determining whether the cells of the inner cell mass express the transgene.

21. A method according to Claim 20 wherein the transgene is expressed substantially only in cells of the inner cell mass.

22. A method according to any of Claims 19 to 21 wherein the transgene

codes for a selectable marker, and wherein cells not expressing the selectable marker can be selectively killed or ablated in culture.

23. A method according to any of Claims 19 to 21 wherein the transgene codes for a reporter marker which is excited when illuminated with light of a certain wavelength, and wherein cells expressing the reporter marker can be identified by their emission in the presence of the light.

24. An assay for the effect of a factor on a nucleus of a somatic cell, comprising:-

introducing the nucleus into an enucleated cell, wherein the nucleus comprises a marker that is differentially expressed in (a) a desired stem cell, and (b) a cell other than the desired stem cell;

introducing the factor into the cell;

maintaining the cell in *in vitro* culture; and

determining whether the cell or any of its daughter cells are expressing the marker, whereby expression of the marker indicates the somatic cell nucleus has been reprogrammed to be a nucleus of the desired stem cell.

25. An assay according to Claim 24, wherein the marker is differentially expressed in (a) multipotential stem cells, and (b) cells other than multipotential stem cells.

26. An assay according to Claim 24 or 25, for assay of a library of factors.

27. An assay according to Claim 26 for assay of a library of factors obtainable by expression of a library of cDNAs obtained from mRNA extracted from the cytoplasm of an oocyte.

28. An assay according to any of Claims 24 to 27, for identifying a factor that induces reprogramming of the nucleus of a somatic cell into the nucleus of a multipotential cell.

29. An assay according to any of Claims 24 to 27, for identifying a factor that induces reprogramming of the nucleus of a somatic cell into the nucleus of an embryonic stem cell.

30. An assay to determine whether an embryo or embryonic structure contains embryonic stem cells, comprising:-

introducing into an enucleated oocyte the nucleus of a somatic cell comprising a marker differentially expressed in (a) embryonic stem cells, and (b) cells other than embryonic stem cells;

obtaining therefrom an embryo or embryonic structure; and

determining whether cells of the embryo or embryonic structure express the marker.

31. An assay according to Claim 30, comprising introducing into the somatic cell a transgene containing a sequence coding for the marker under control of a promoter activated substantially only in embryonic stem cells.

32. An assay according to Claim 31 wherein the promoter is activated in cells that express the OCT 4 gene.

33. An assay according to Claim 32 wherein the promoter is the OCT 4 promoter.

34. An assay according to any of Claims 31 to 33, wherein the transgene also contains a sequence coding for a second marker under control of a

promoter activated in substantially all cells, and the assay comprises determining whether the transgene has been stably transfected into the nucleus by determining whether the second marker, is being expressed prior to introduction of the nucleus into the enucleated oocyte.

35. An assay according to Claim 34, wherein the promoter for the second marker is the beta-actin promoter.

36. An embryonic stem cell obtainable according to the method of any of claims 1 to 23.

37. A human embryonic stem cell derived from a non-human oocyte.

38. A human embryonic stem cell derived from a non-human mammalian oocyte.

39. A human embryonic stem cell according to Claim 38, derived from a pig or cow oocyte.

40. A human embryonic stem cell derived from a human oocyte.

41. A stem cell for transplantation into a first human and being immunologically compatible with the first human, derived from an oocyte of a different human.

42. An embryonic stem cell of a first mammalian species, derived from an oocyte of a species other than the first species, and comprising a transgene coding for a selectable marker which is substantially only expressed in the embryonic stem cell.

43. A method of obtaining a human embryonic stem cell, suitable for derivation of human stem cells or human somatic cells for

transplantation into a first human individual, comprising introducing into an oocyte other than of that first individual the nucleus from a somatic cell of that first individual.

44. A method according to Claim 43, wherein the human embryonic stem cell contains a transgene coding for a selectable marker which is substantially only expressed in the human embryonic stem cell.

45. A multipotential cell that expresses a transgene which identifies its undifferentiated state, which cell is genetically identical, apart from the presence of the transgene, to an adult animal.

46. A method of reprogramming a somatic nucleus into a pluripotent nucleus, comprising:-

identifying a factor that induces reprogramming of the nucleus, by carrying out the assay according to any of Claims 24 to 29;

obtaining a somatic cell nucleus;

obtaining an enucleated cell;

transferring the somatic cell nucleus into the enucleated cell to form a recombinant cell;

culturing the recombinant cell *in vitro* in the presence of the factor.

47. A method of reprogramming a somatic nucleus into a totipotent nucleus, comprising:-

identifying a factor that induces reprogramming of the nucleus, by carrying out the assay according to any of Claims 24 to 29;

obtaining a somatic cell, and culturing it in the presence of the factor;

obtaining an enucleated cell;

transferring the nucleus of the somatic cell into the enucleated cell to form a recombinant cell;

culturing the recombinant cell *in vitro*.

48. A method according to Claim 46 or 47, further comprising:-

introducing into the somatic cell a gene coding for a marker which is differentially expressed in (a) desired totipotent cells, and (b) cells other than desired totipotent cells;

transferring the nucleus of the somatic cell into an enucleated oocyte; and

determining whether cells of an embryo obtained therefrom are expressing the marker.

FIG. 1

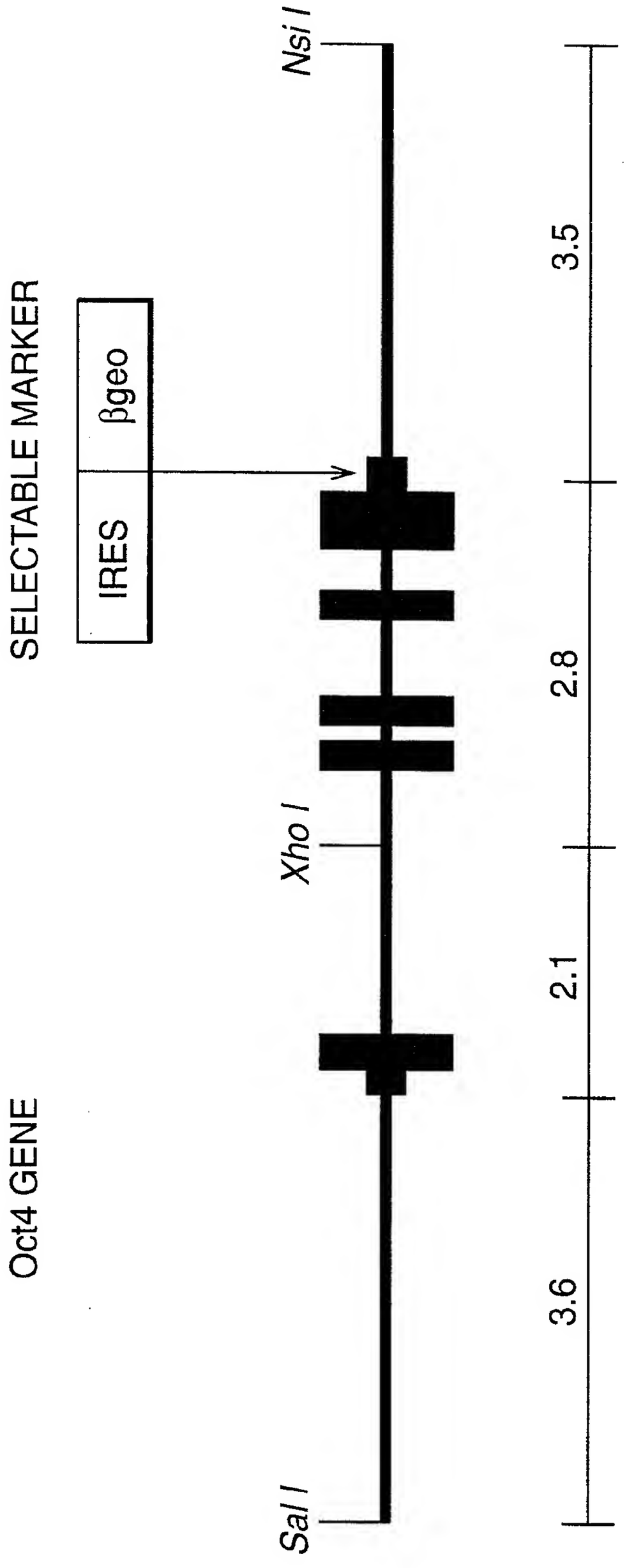
Nsi I
SELECTABLE MARKER
βgeo
IRES
Xho I
Oct4 GENE
Sal I
3.5
2.8
2.1
3.6

FIG. 2

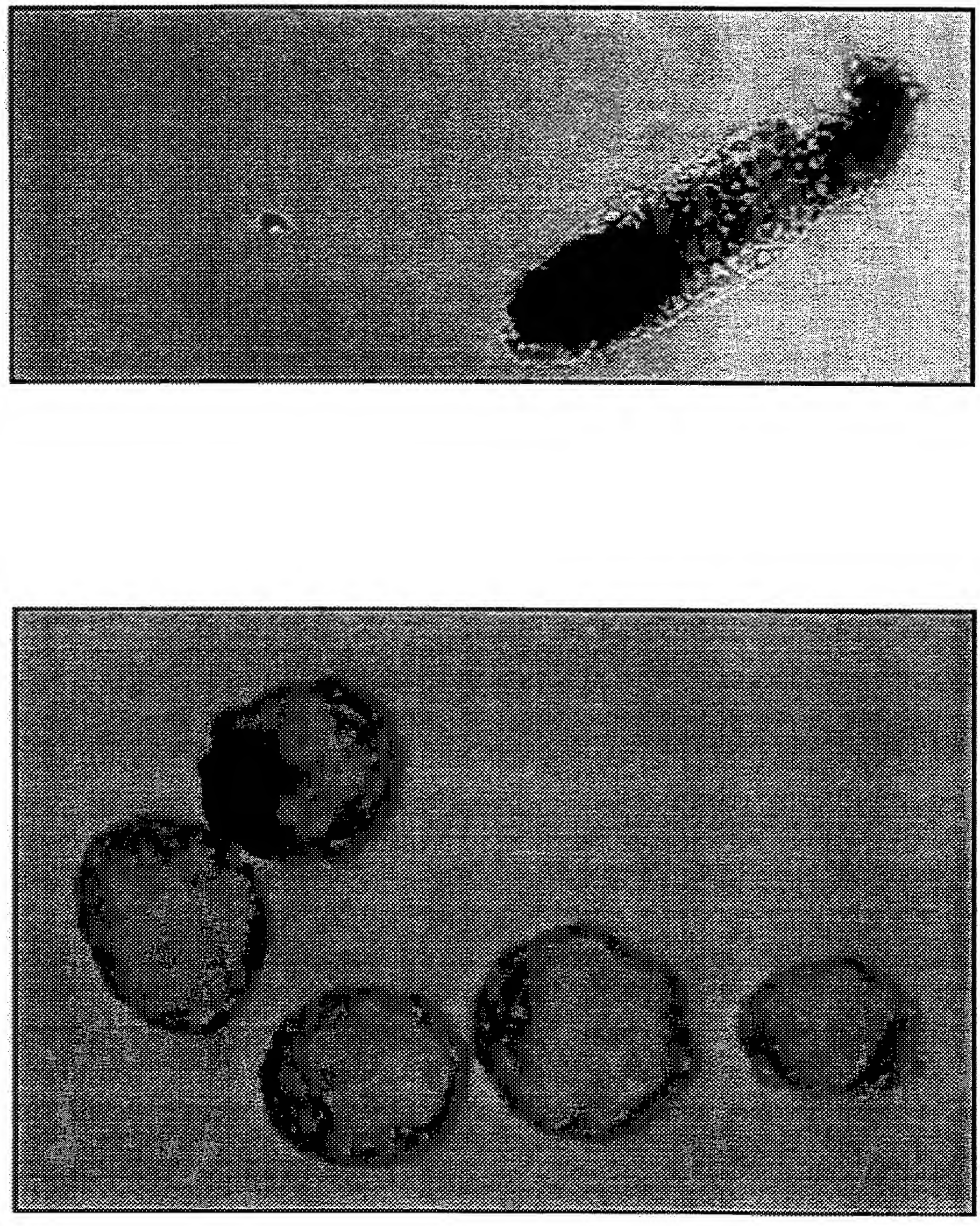

FIG. 3

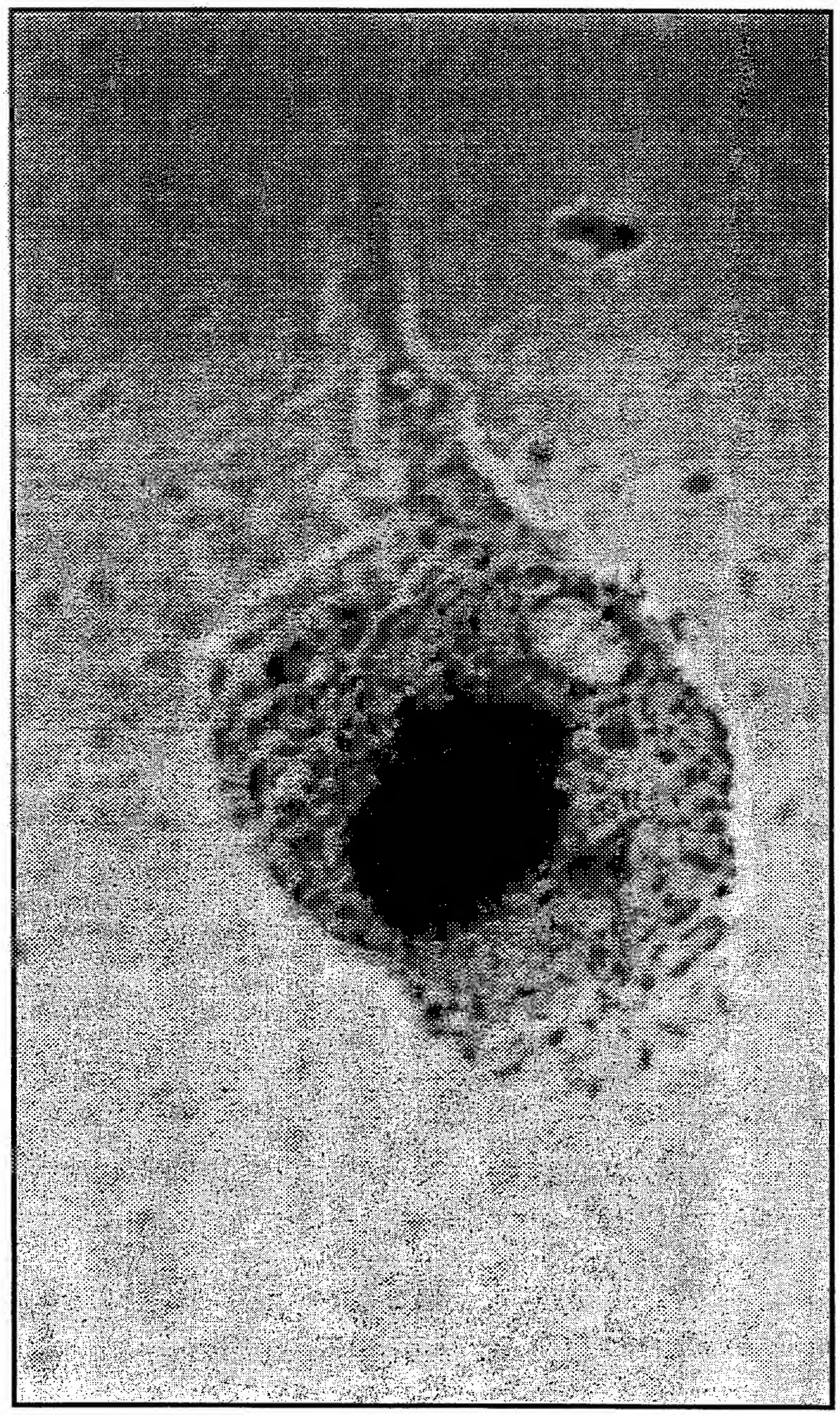

FIG. 4

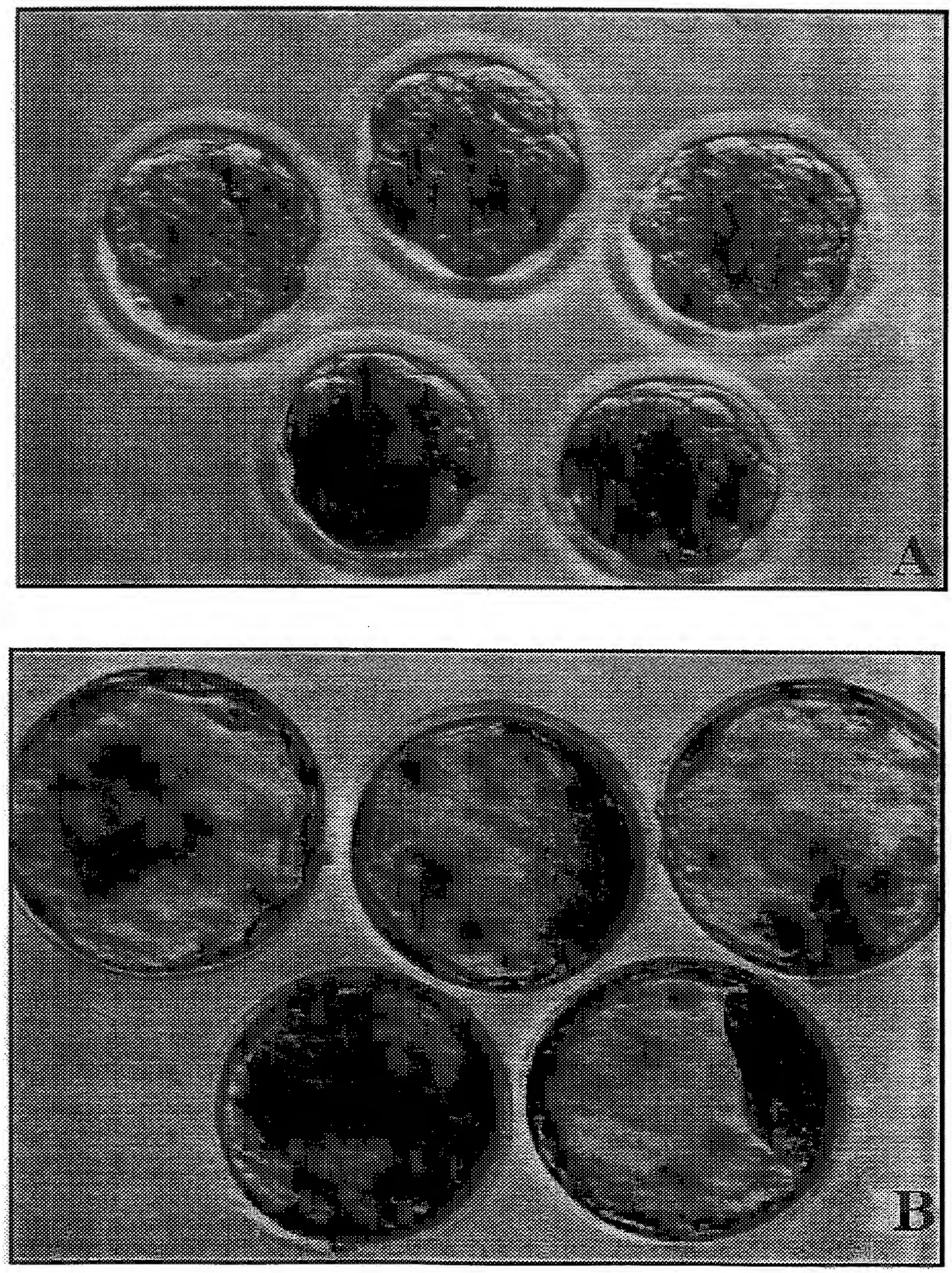

FIG. 5